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Conversion of Averufin into Aflatoxins by *Aspergillus parasiticus*†

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ABSTRACT: Resting mycelium of *Aspergillus parasiticus* ATCC 15517 efficiently converted averufin ($C_{20}H_{16}O_7$) into aflatoxin B_1, B_2, and G_1 with only minor dilution. Autoclaved mycelium did not catalyze this conversion. The removal of averufin from the medium and the incorporation into aflatoxin are both time dependent and detectable within 2 hr of incubation. [^{14}C]-Acetate-labeled averufin for the above experiments was prepared using a mutant of *A. parasiticus* which elaborates large amounts of averufin, but very little aflatoxin. Traces of averufin were also found in wild-type mycelium. The results indicate that the C_{20} polyketide-derived polyhydroxyanthraquinone averufin, or a metabolite closely related to it, is an intermediate in aflatoxin biosynthesis. A possible pathway for the conversion of averufin into aflatoxin B_1 is discussed.

The aflatoxins (Büchi and Rae, 1969; Figure 1) are a closely related group of secondary metabolites produced by certain *Aspergilli*. They can act as acute toxins and as chemical carcinogens in many animal species. They have been found as contaminants in various animal feeds and human foodstuffs, and have been implicated in the etiology of human liver cancer in certain parts of the world (Kraybill and Shapiro, 1969; Goldblatt, 1972). Two of the more readily noticeable acute *in vivo* effects of aflatoxin B_1 (or a metabolite derived from it; Moulé and Frayssinet, 1972), the most toxic and usually also the most prominent of the aflatoxins, are inhibition of DNA and DNA-directed RNA synthesis (reviewed by Wogan, 1969).

Biosynthesis of the aflatoxins has also attracted considerable attention (Mateles and Wogan, 1967; Lillehoj *et al.*, 1970; Detroy *et al.*, 1971). Incorporation experiments (Donkersloot *et al.*, 1968; Hsieh and Mateles, 1970), followed by degradative studies (Biollaz *et al.*, 1968a), have revealed that aflatoxin B_1 can be derived efficiently from acetate, and that methionine can act as a methyl donor for the methoxymethyl group (Figure 1). Based upon these findings, Biollaz *et al.* (1968b) proposed a scheme in which a C_{18} polyketide-derived polyhydroxynaphthacene would be an early intermediate. This compound would undergo endo oxygenation and yield, after rearrangement and isomerization, a difuroanthraquinone of the versicolorin series. Thomas (1965) had already speculated earlier that averufin or its derivative could be involved in aflatoxin biogenesis. Such an intermediate would, after oxidative removal of a quinone carbon as CO_2 and recyclization, yield a difuroxanthone of the sterigmatocystin type. The latter would subsequently undergo another loss of CO_2 and yield aflatoxin B_1 (reviewed by Biollaz *et al.*, 1970). This hypothesis was based, in part, upon the co-occurrence of aflatoxins and representatives of both the difuroanthraquinone series (versicolorin C; Heathcote and Dutton, 1969) and the difuroxanthone series (*O*-methylsterigmatocystin and aspertoxin; Burkhardt and Forgacs, 1968; Rodricks *et al.*, 1968; Waiss *et al.*, 1968) in certain members of the *Aspergillus flavus* group. Experimental support for a difuroxanthone precursor in aflatoxin biosynthesis was provided by Elsworthy *et al.* (1970), who showed that 5-hydroxydihydrosterigmatocystin could be incorporated into aflatoxins B_2 and G_2 (2% conversion) and by Hsieh *et al.* (1973), who showed that sterigmatocystin could be incorporated into aflatoxin B_1 (20% conversion). Experimental evidence for the early events in the biogenetic scheme proposed by Biollaz *et al.* (1970) has not been obtained so far.

To identify any early intermediates, a search was initiated for mutants blocked in aflatoxin synthesis. One such mutant produced large quantities of the polyhydroxyanthraquinone

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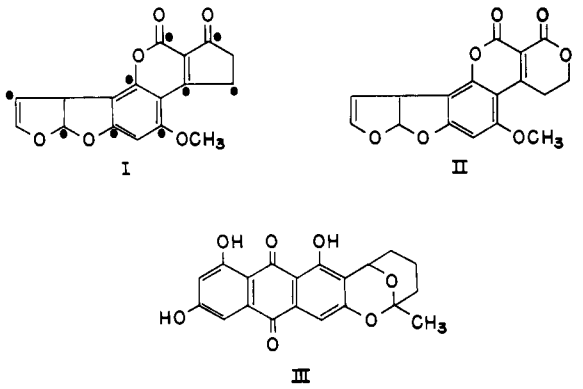

FIGURE 1: Structure of aflatoxins B_1 (I), G_1 (II), and averufin (III). The labeling pattern of aflatoxin B_1 with $CH_3\dot{C}OOH$ as precursor is also indicated (Biollaz *et al.*, 1968a). Aflatoxins B_2 and G_2 are congeners of B_1 and G_1, respectively, with a reduced bisfurano moiety.

averufin ($C_{20}H_{16}O_7$; Figure 1, Donkersloot *et al.*, 1972), and this mutant could therefore be used to prepare [^{14}C]averufin (Hsieh *et al.*, 1972).

By using the labeled compound, it was now possible to test averufin for a precursor role in the biosynthesis of the aflatoxins. In our study, we found that averufin is rapidly taken up by resting mycelium of the parent fungus and then converted in good yield into aflatoxin B_1, B_2, and G_1 with only minor dilution.

Materials and Methods

Strains and Culture Methods. Wild-type *Aspergillus parasiticus* ATCC 15517 and its nitrosoguanidine derived averufin-producing mutant ATCC 24551 (formerly called W49, Donkersloot *et al.*, 1972) were grown and the conidia harvested and stored as described by Donkersloot and Mateles (1968).

About 10^6 conidia were inoculated into 100 ml of a glucose–ammonium sulfate–salts medium (Adye and Mateles, 1964) in a 500-ml indented flask. The mutant culture was supplemented with 0.1% yeast extract to enhance averufin production. After incubation on a rotary shaker at 30° for 48 (parent) or 72 hr (mutant), the mycelial pellets were collected on cheesecloth

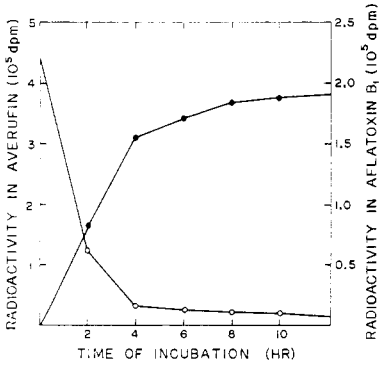

FIGURE 2: Time course of [^{14}C]averufin (○) conversion into aflatoxin B_1 (●) by wild-type *A. parasiticus*. [^{14}C]Averufin (0.22 μCi, 0.19 μmol) was added to seven resting cell suspensions, and each culture was incubated for the period shown. Averufin and aflatoxin B_1 were isolated and purified and their radioactivity was determined as described in the text. Each value shown represents the average of two flasks.

TABLE 1: Incorporation of [1-^{14}C]Acetate and [^{14}C]Averufin into Aflatoxin B_1 by *Aspergillus parasiticus* ATCC 15517.[a]

Precursor	μCi Added[b]	10^4 dpm in B_1	Resting Cells
Acetate	7.9	7.4 (0.4%)[c]	Not autoclaved
Acetate	7.9	0	Autoclaved
Averufin	0.18	3.3 (8.4%)	Not autoclaved
Averufin	0.18	0	Autoclaved

[a] The resting cell suspension was incubated for 20 hr (30°, 150 rpm). [b] The specific activities of the precursors were: acetate, 0.13 Ci/mol; averufin, 0.53 Ci/mol. [c] Per cent of added radioactivity retained in purified aflatoxin B_1.

and washed with N-free resting cell medium[1] (RC medium, Adye and Mateles, 1964).

[1-^{14}C]Acetate-labeled averufin was prepared by the method of Hsieh *et al.* (1972). Five grams (wet weight) of mutant mycelial pellets was added to 100 ml of RC medium and the culture incubated (30°, 150 rpm). At the beginning and at 1-hr intervals thereafter, 0.1-mCi portions of sodium [1-^{14}C]-acetate (57 Ci/mol; New England Nuclear) were added (0.5 mCi total). The pellets were collected after 5 hr and extracted with acetone. The averufin in the extract was concentrated under vacuum and purified by thin-layer chromatography (tlc) on ChromAR 500 sheets (Mallinckrodt Chemical Works) in unequilibrated tanks using the following solvent systems (volumetric ratios) in succession: chloroform–acetone–*n*-hexane (CAH, 85:15:20), benzene–ethyl alcohol–water (BEW, 46:35:19), and benzene–petroleum ether–acetone (BPA, 90:8:2). After each tlc run, the averufin-containing zone (R_F values 0.8, 0.8, and 0.25, respectively, in the three solvents used) was cut out and the pigment eluted with acetone and concentrated. Averufin thus purified had the same ultraviolet–visible spectrum as an authentic sample and was free from aflatoxins and other impurities as judged by tlc and the coincidence of the averufin spot with the ^{14}C image on an autoradiogram. The concentration of averufin was determined by measuring its absorbance at 453 nm (ε 10,500; Donkersloot *et al.*, 1972).

Autoradiography. To prepare an autoradiogram, a tlc plate was covered with No-Screen Medical X-Ray film (Eastman Kodak Co.) and stored in darkness for 2–7 days. After developing and fixing, the autoradiogram was superimposed on the tlc plate and examined under long-wavelength ultraviolet (uv) light.

Incorporation Studies. RC medium (9.8 ml) containing 90 μmol of glucose was slowly added to 0.2 ml of [^{14}C]averufin in acetone (see Figure 2 and Tables I and II for details) in a 50-ml indented flask. By this procedure, 68% of the [^{14}C]-averufin remained in the aqueous medium. One gram of mycelial pellets was then added to each flask. Controls containing [1-^{14}C]acetate instead of averufin were used to determine the synthetic activity of the cells. Acetone at this concentration (2% v/v) reduced the acetate incorporation by 37%. Other controls contained autoclaved cells and either [1-^{14}C]-acetate or [^{14}C]averufin. After the incubation (20 hr, 30°, 150

[1] Abbreviations used are: RC medium, N-free resting cell medium; CAH, chloroform–acetone–*n*-hexane; BEW, benzene–ethyl alcohol–water; BPA, benzene–petroleum ether–acetone; RSA, relative specific activity; EIW, ethyl acetate–isopropyl alcohol–water.

rpm), the aflatoxins were extracted from the broth with chloroform. In certain experiments, a known quantity of mixed aflatoxins (Calbiochem) was added to each sample as a carrier.

To study the kinetics of the conversion of averufin into aflatoxin B_1, separate cultures were incubated with [^{14}C]-averufin (0.22 μCi, 0.19 μmol) for periods of 0, 2, 4, 6, 8, 10, and 12 hr, as described above. Afterwards, the mycelium was separated from the broth and extracted with acetone, and the broth was extracted with chloroform. The extracts were combined, evaporated under vacuum, and taken up in 0.2 ml of chloroform. Ten microliters was applied onto Adsorbosil-1 plates (Applied Science Lab) for two-dimensional tlc (benzene–acetone (96:4), followed by CAH). The aflatoxin B_1 and averufin spots were scraped directly into scintillation fluid.

The results of these incorporation experiments are given as per cent conversion and as relative specific activity (RSA) values. The conversion percentage is the radioactivity (disintegrations per minute) in the purified product divided by the radioactivity added as precursor. The RSA is the ratio between the specific activity (curies per mole) of the purified product and the specific activity of the precursor (Hsieh and Mateles, 1970).

Purification of Aflatoxins. Aflatoxins were separated from residual averufin and other impurities by tlc on ChromAR 500 sheets using two solvent systems in succession: ethyl acetate–isopropyl alcohol–water (10:2:1, EIW) and BPA. In these systems, the aflatoxin group appeared as a compact band with R_F values of 0.91 and 0.04, respectively, whereas averufin had R_F values of 0.97 and 0.25. After elution with chloroform, aflatoxins B_1, B_2, G_1, and G_2 were separated and further purified on Adsorbosil-1 plates with CAH and EIW as solvents. Aflatoxins were identified by their blue (B_1 and B_2) or green (G_1 and G_2) fluorescence under long-wavelength uv light and by comparison with standard aflatoxins applied on the same plate. Aflatoxins were measured by uv spectrophotometry at 360 nm (Nabney and Nesbitt, 1964). Autoradiography was periodically used to determine the radiochemical purity. In one instance, aflatoxin B_1 was further purified with two tlc systems (CAH, EIW). The specific radioactivity of aflatoxin B_1 remained constant after each of these purifications.

Co-occurrence of Averufin and Aflatoxins. Two methods were used to test for the co-occurrence of averufin and aflatoxins in the wild-type strain. In the first method, the broth and the pellets of a 48-hr culture were separated and extracted with chloroform and acetone, respectively. Aflatoxins and averufin were then separated and purified by the methods described above. In the second (isotope dilution) method, the culture was incubated with 90 μmol of [1-^{14}C]acetate (0.17 Ci/mol) for 10 hr (150 rpm). Sixty micrograms of nonradioactive averufin was added as carrier before the extraction. After isolation and purification, the radioactivity of averufin and aflatoxins was determined.

Radioactivity Measurements. Samples were pipeted or scraped into glass vials containing 15 ml of a scintillation fluid and counted in a Packard Model 2425 Tri-Carb liquid scintillation spectrometer. Counting efficiency, as determined by the automatic external standardization method, was in the range of 84–88% for all samples measured.

Results

Conversion of Averufin into Aflatoxins. The incorporation of [^{14}C]averufin and [1-^{14}C]acetate into aflatoxin B_1 by wild-type *A. parasiticus* is indicated in Table I. After extensive purification, aflatoxin B_1 had retained 8.4% of the radioactivity from [^{14}C]averufin. With acetate (6 mM), the conversion was much lower (0.4%). Acetate is known to be completely metabolized under the conditions used (Hsieh and Mateles, 1970, 1971). Because the concentration of averufin was low (0.03 mM), and because the conversion was evident within 2 hr (see below), it appears quite unlikely that the observed incorporation was due to previous degradation to acetate. Actual conversion of averufin must have been more than 10%, as some aflatoxin B_1 is inevitably lost during the repeated tlc purifications. Special care was taken to free aflatoxin B_1 from averufin. The result obtained with autoclaved mycelium validates the purification procedure and shows that enzymes were responsible for the conversion.

TABLE II: Specific Radioactivity and Relative Specific Activity[a] of Aflatoxin B_1, Derived from [^{14}C]Averufin or [1-^{14}C]-Acetate.[b]

Precursor	Specific Radioact. (Ci/mol)	
	Precursor	Aflatoxin B_1
[^{14}C]Averufin	0.293	0.200 (0.67)[a]
[1-^{14}C]Acetate	0.167	0.062 (0.37)[a]

[a] RSA (relative specific activity) = specific radioactivity (product)/specific radioactivity (precursor). [b] Flasks containing 10 ml of resting cell culture and 1.5 μmol of [^{14}C]averufin or 30 μmol of [1-^{14}C]acetate were incubated for 20 hr. Aflatoxin B_1 from each flask was purified and its specific radioactivity determined. The values shown are the average of two experiments.

The time course of averufin incorporation into aflatoxins is shown in Figure 2. The result reveals that the conversion of averufin to aflatoxin B_1 was time dependent. Under the conditions used, more than 80% of the averufin was taken up by the cells within 4 hr and converted into metabolites such as aflatoxin B_1.

The time-dependent conversion of averufin to aflatoxin B_1 was again evident on the autoradiogram (Figure 3) prepared from a tlc plate of samples collected in the time course experiment. After 2 hr, radioactivity was found in aflatoxins B_1, G_1, and B_2 and in several other, presently unknown, metabolites with lower R_F values. The radioactivity in some of these compounds (*e.g.* the yellow pigment X) later decreased, whereas that in aflatoxin B_1 increased. These compounds might therefore be intermediates in the conversion of averufin into aflatoxin. They could also be early intermediates in the catabolism of averufin. Further characterization of these metabolites is in progress.

To determine the RSA of aflatoxin B_1 derived from [^{14}C]-averufin and from [1-^{14}C]acetate, an experiment was done in which no carrier was added before the labeled aflatoxin B_1 was isolated and purified. The results (Table II) indicate that averufin was incorporated into aflatoxin B_1 with only minor dilution (RSA = 0.67), despite the fairly low concentration (0.15 mM) at which it was offered. The theoretical maximum RSA for this conversion is 0.9, as ten carboxyl positions from acetate are incorporated into averufin but only nine into aflatoxin B_1 (Biollaz *et al.*, 1968a). This maximum RSA value could only be obtained if there were no carry-over of aflatoxins from the primary culture, and no endogenous synthesis of aflatoxins from glucose during the actual incorporation of averufin.

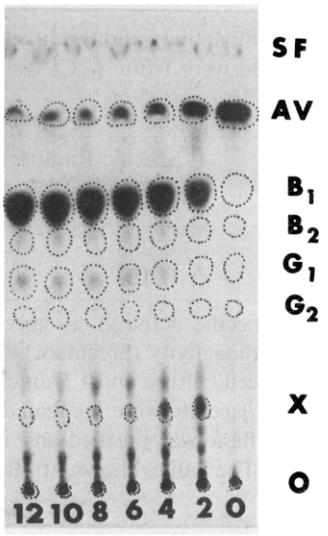

FIGURE 3: Time-dependent conversion of [^{14}C]averufin into other metabolites. Extracts of cultures harvested after 0–12-hr incubation (see Figure 2) were chromatographed on Adsorbosil-1 with chloroform–acetone–*n*-hexane as solvent (85:15:20, v/v/v). An autoradiogram was prepared from and superimposed on this tlc plate. Dotted lines indicate the location of fluorescent spots on the tlc plate: SF, solvent front; AV, averufin; B_1, B_2, G_1, and G_2, individual aflatoxins; X, unknown yellow pigment; and O, origin.

With acetate, the RSA was 0.37 (theoretical maximum RSA = 9). This lower value was due to the metabolic consumption of acetate, the dilution of radioactive acetate by unlabeled, glucose-derived acetate during the 20-hr incubation period, and the carry-over of aflatoxins from the primary culture (Hsieh and Mateles, 1970, 1971). These results confirm that averufin was converted into aflatoxins without the intermediary formation of acetate.

Coexistence of Averufin and Aflatoxin. Averufin could be detected in 48-hr mycelium of wild-type *A. parasiticus* (Table III). Aflatoxins are commonly found in both the mycelium and the spent broth, but averufin is present almost exclusively in the mycelium. The amount of aflatoxin (in the broth and the mycelium) was about 43 times greater than that of averufin. The co-occurrence of averufin and aflatoxins was confirmed by the incorporation of [1-^{14}C]acetate into the two metabolites (Table IV). These results show that the parent strain elaborates small quantities of averufin and large amounts of aflatoxins. Preliminary experiments also indicate averufin synthesis by the other aflatoxin-producing strains that were tested.

TABLE III: Production of Averufin and Aflatoxins by *A. parasiticus* ATCC 15517.[a]

Flask No.	Aflatoxins in Broth	Aflatoxins in Pellets	Total Aflatoxins	Averufin in Pellets
1	3.39[b]	0.94[b]	4.33[b]	0.08[b]
2	3.48	0.80	4.28	0.11
Av	3.44	0.87	4.31	0.10

[a] A 1-ml spore suspension (10^6 conidia) was inoculated into 100 ml of growth (AM) medium and the culture incubated at 30° for 48 hr with shaking. Aflatoxins and averufin were then isolated and purified and their concentration was determined by spectrophotometry. [b] μmol per 100 ml of medium.

TABLE IV: Incorporation of Radioactivity from [1-^{14}C]Acetate into Averufin and Aflatoxins by *A. parasiticus* ATCC 15517.[a]

Flask No.	Radioactivity (10^5 dpm)	
	Aflatoxins	Averufin
1	4.60 (1.40)[b]	1.54 (0.47)[b]
2	4.10 (1.24)	1.12 (0.34)
Av	4.35 (1.32)	1.33 (0.41)

[a] Each 10-ml resting cell culture was supplemented with 90 μmol of [1-^{14}C]acetate (0.17 Ci/mol) and incubated for 10 hr. Averufin and aflatoxins were isolated and purified as described in the Materials and Methods section. [b] Per cent of added radioactivity retained.

Discussion

The results presented show that *A. parasiticus* readily metabolizes averufin to aflatoxins. This conversion is rapid and time dependent, and occurs in good yield with only minor dilution. We suspected that a relatively insoluble and apolar compound such as averufin might not be taken up, but the resting cell system had little difficulty in metabolizing averufin. This acetone containing system thus provides a useful method for testing other possible intermediates.

The co-occurrence of aflatoxin and averufin supports our assumption that C_{20} polyketide-derived compounds are involved in aflatoxin biogenesis. Until now, averufin has not been reported as a cometabolite of the aflatoxins, although some closely related compounds have been detected (Heathcote and Dutton, 1969; Lee *et al.*, 1971).

We infer from the present results that averufin, or a closely related metabolite, is an intermediate in aflatoxin biosynthesis. Averufin fulfills two of the postulated requirements for an intermediate: *i.e.*, it is excreted by a mutant blocked in aflatoxin synthesis, and it is converted into aflatoxin by the parent (Umbarger and Davis, 1962). Studies with purified enzymes are needed to prove or disprove that averufin is on the direct pathway.

Averufin was first proposed as a possible intermediate in the biosynthesis of versicolorin, sterigmatocystin, and aflatoxin by Thomas in 1965 (for a revised scheme, see Mateles and Wogan, 1967). In this scheme, the C_4 bisfuran moiety of these metabolites is derived from the C_6 side chain of averufin. But the experimentally found labeling pattern of the bisfuran group in aflatoxin B_1 (Biollaz *et al.*, 1968a, Figure 1) refuted this hypothesis. Both degradative and ^{13}C nuclear magnetic resonance (nmr) studies on sterigmatocystin (Holker and Mulheirn, 1968; Tanabe *et al.*, 1970) have confirmed the bisfuran labeling pattern. Recently, Thomas has proposed a new mechanism for the conversion of the C_6 side chain of averufin into the bisfuran group (Moss, 1972). This proposal is consonant with all reported experimental findings.

Therefore, our results lead to the working hypothesis that aflatoxins arise through the condensation of one acetyl-coenzyme A and nine malonyl-coenzyme A molecules to form a C_{20} polyketide. This unstable intermediate is modified either during or after its formation and cyclization, and appears as a C_{20} polyhydroxyanthraquinone in the cytoplasm. Subsequent steps leading to a sterigmatocystin type compound could occur according to the recent Thomas' scheme, but need to be proven experimentally. Good evidence is available for the final

conversion of 5-hydroxysterigmatocystin and sterigmatocystin into aflatoxins (Elsworthy *et al.*, 1970; Hsieh *et al.*, 1973).

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